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Fatty Acid and Wax Biosynthesis in Susceptible and Triallate-Resistant *Avena fatua* L.

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Abstract: The recent characterization of triallate-resistant lines of wild oat (*Avena fatua* L.) deficient in triallate sulfoxidation provides an experimental system to investigate and differentiate the effects of triallate and triallate sulfoxide on wax and lipid biosynthesis. Greenhouse applications of triallate dramatically reduced epicuticular wax deposition in susceptible (S) but not resistant (R) wild oats. Triallate treatment had no effect on in-vivo concentrations of C_{12} to C_{26} fatty acids and fatty alcohols in R plants, while elongated fatty acid fractions ($C > 18$) were significantly reduced in S plants. In contrast, treatment with triallate sulfoxide reduced in-vivo concentrations of elongated fatty acids equally in R and S, supporting the hypothesis that triallate sulfoxide is more inhibitory than triallate towards fatty acid elongases. Although de-novo synthesis of short-chain fatty acids was not affected by triallate or triallate sulfoxide in R or S plants, synthesis of elongated fatty acid fractions was dramatically reduced in S plants by triallate. Fatty acid biosynthesis in R and S plants was equally sensitive to triallate sulfoxide. The results support the idea that in-vivo triallate sulfoxidation is necessary for herbicidal activity, and confirm that reduced rates of triallate sulfoxidation confer resistance in R wild oats.

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1 INTRODUCTION

Triallate is a selective pre-emergence thiocarbamate herbicide used to control wild oats (*Avena fatua* L.) in barley (*Hordeum vulgare* L.) and tolerant cultivars of spring wheat (*Triticum aestivum* L.). Extensive use of triallate has selected for resistant (R) wild oat populations that are 12- to 20-fold more tolerant to the herbicide than susceptible (S) populations.[1,2] Recent studies showed that resistance was due to a decreased rate of activation (sulfoxidation) of the proherbicide triallate, resulting in reduced in-vivo concentrations of the phytotoxic triallate sulfoxide.[3] Characterization of this resistance mechanism allowed us to conduct new studies addressing triallate's mode of herbicidal action and the role that an altered metabolic pathway may play in triallate's phytotoxicity.

Thiocarbamate herbicides inhibit the biosynthesis of surface lipids and epicuticular waxes, although it is not clear if this inhibition is the sole mechanism of herbicidal action.[4,5] Epicuticular waxes are synthesized by condensation of 16- and 18-carbon fatty acids with malonyl-CoA by acyl-coenzyme A fatty acid elongases in NADPH-dependent reactions.[4] Since inhibition of wax biosynthesis could be caused by inhibition of elongation reactions or decreased formation of the C_{12} to C_{18} precursors, much of the research investigating thiocarbamate action in plants has been directed toward studying fatty acid biosynthesis and the subsequent elongation reactions. Incorporation of [^{14}C]acetate into total lipids was reduced 43–88% by the thiocarbamate diallate in *Arachis hypogaea* L. leaf discs, indicating that inhibition of wax precursor biosynthesis could be responsible for reduced epicuticular wax deposition.[6]

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However, analysis of lipid fractions from triallate-treated pea (*Pisum sativum* L.) seedlings showed that only elongated C_{20}, C_{22} and C_{24} fractions were reduced, while biosynthesis of palmitate (C_{16}) and stearate (C_{18}) was not affected.[4,7,8] These and other studies support the idea that the primary inhibition of lipid biosynthesis is at the level of fatty acid elongation, and that general fatty acid biosynthesis is not affected.

The first step of thiocarbamate metabolism in plants is an enzyme-mediated sulfoxidation reaction yielding sulfoxide derivatives which most likely have greater toxicity than their respective proherbicides,[9] although increased toxicity was not seen for all members of this herbicide class.[10] Several thiocarbamate sulfoxides have been shown to bind covalently sulfhydryl-containing molecules and thus have been classified as carbamoylating agents.[11,12] Barrett and Harwood[8] showed that the sulfoxide derivative of pebulate inhibited the formation of very long-chain fatty acids in peas to a greater extent than the parent herbicide. Likewise, triallate and diallate inhibited fatty acid elongation but not palmitate and stearate synthesis in whole pea plants, and not in isolated pea microsomes.[7] Abulnaja and Harwood[7] proposed that the *S*-chloroallyl thiocarbamate sulfoxide derivatives (which are not formed in microsomal fractions) selectively inhibited fatty acid elongases without affecting synthesis of short-chain fatty acids. Thus, the thiocarbamate sulfoxide derivatives are most likely the herbicidally active molecules *in planta*.

Previous studies investigating the effects of thiocarbamates and their sulfoxide derivatives on whole plant fatty acid biosynthesis have not been able to differentiate between parent molecule and sulfoxide effects, because of rapid in-vivo sulfoxidation reactions. However, the dramatically reduced rates of sulfoxidation in triallate-resistant wild oat populations offers an excellent model system to make such comparisons.[3] The specific objectives of these studies were to compare the effects of triallate and triallate sulfoxide treatment on epicuticular wax deposition, free fatty acid pool sizes, and de-novo fatty acid biosynthesis in R and S wild oats.

2 EXPERIMENTAL METHODS

2.1 Plant material

S wild oat seeds were collected from greenhouse-grown plants of the dormant and nondormant inbred lines AN265 and SH430,[13] respectively, grown in 1993. In addition, two field accessions were tested to compare lipid profiles from several different wild oat accessions. Results from the SH430 experiments were representative of all S lines and collections tested and are reported here.

Wild oat seeds were collected in 1993 from fields near Fairfield, MT that had been treated with triallate for several consecutive years and contained a high proportion of triallate-resistant individuals.[14] One of these collections, FG93R22, had the highest proportion of R individuals and was used to develop an inbred R line. Approximately 200 FG93R22 plants surviving greenhouse treatment with 1·1 kg ha^{-1} triallate were grown to maturity, selfed, and their progeny subjected to an indentical herbicide treatment. The surviving individuals (about 1000 plants) were again grown, selfed, and their progeny used for the experiments reported here. R and S seeds were dehulled (lemma and palea removed by hand) to aid in surface sterilization and enhance germination.

2.2 Synthesis of triallate sulfoxide

Triallate sulfoxide was synthesized from technical grade triallate (Monsanto, 98·9%) using the method of Schuphan *et al.*[15] with the following modifications. An emulsion of 1000 mg $litre^{-1}$ triallate was made in distilled deionized water (1 ml) containing octyl phenoxy polyethoxyethanol surfactant ('Triton' X-100, Sigma cat. no. T9284; 70 g $litre^{-1}$), and $1{\cdot}67 \times 10^2$ Bq [1-^{14}C]triallate (Monsanto, sp. act. $3{\cdot}42 \times 10^6$ Bq mg^{-1}) was added to the reaction mixture to monitor the reaction progress radioisotopically. *Meta*-chloroperoxybenzoic acid (Sigma cat. no. C9416) was added in a 1·4-fold molar excess, the mixture vortexed for 30 s and then incubated on ice for 1 h. Upon completion of the reaction (>99% conversion as determined by HPLC analysis; see below), the emulsion was diluted to the desired herbicide concentration in deionized water containing 70 g $litre^{-1}$ surfactant and used within 10 min.

2.3 HPLC analyses

HPLC conditions for monitoring the triallate sulfoxidation reaction used a C18 reverse phase column in $4{\cdot}6 \times 250$ mm format. The mobile phase consisted of water + acetonitrile (95 + 5 by volume) on injection, which was ramped linearly to 50 + 50 by volume over 10 min, ramped to 100% acetonitrile over the next 2 min, and held for an additional 5 min. The gradient programmer (Isco model 2369 gradient programmer, Isco, Inc.) and HPLC pump apparatus (Isco model 2350 HPLC pump, Isco, Inc.) delivered solvent at 2·0 ml min^{-1} and separated triallate and triallate sulfoxide were detected using an in-line scintillation counter (Beckman model 171 Radioisotope Detector, Beckman Instruments, Inc.). Radioactive peaks were quantified using a dedicated integrator (Shimadzu C-R3A Chromatopac integrator, Shimadzu Corp.).

Chromatographic analyses of radiolabeled free fatty acids were conducted using reverse-phase HPLC as above with the following exceptions. On injection, the mobile phase of water + acetonitrile (30 + 70 by volume) was held for 5 min, then stepped to 100% acetonitrile and held for an additional 15 min. [^{14}C]-labeled fatty acids were identified by comparison of retention times with purchased fatty acid standards (New England Nuclear).

2.4 Epicuticular wax deposition

Caryopses from R and S plants were planted in greenhouse soil mix in 55 × 35 × 10 cm flats. Flats were held in the greenhouse with a 14-h daylength under natural sunlight supplemented with mercury vapor lamps and day/night temperatures of 22/16°C and watered as needed. When the seedlings were approximately 2 cm tall (about seven days after planting), they were treated with 0·1 kg ha^{-1} triallate in 93·5 litre ha^{-1} water using a greenhouse belt sprayer. Five days after treatment, R and S seedlings had doubled in size and a clear 'pinching' effect was visible on treated shoots, delineating leaf tissue present before and after treatment. Leaf tissues were individually harvested and leaf areas determined using a leaf area meter (Li-3100 Leaf Area Meter, Li-Cor, Inc.).

Epicuticular waxes present on R and S seedlings before and after treatment were isolated by ten 1-s dips in HPLC-grade hexane (0·5 ml). Samples were evaporated to dryness, resuspended in a minimal volume of hexane, and converted to trimethylsilyl derivatives using bis(trimethylsilyl)trifluoroacetamide (BSTFA; Aldrich). GC conditions consisted of a 12-m HP-1 capillary column using helium as a carrier gas. After sample injection, oven (HP 5890 Series II Gas Chromatograph, Hewlett Packard, Inc.) temperature was ramped from 150°C to 300°C at 5° min^{-1}. Samples were detected by flame ionization and quantified by comparison to an internal standard of methyl hepadecanoate at 100 ng mg^{-1} of tissue. There were 12 R and 12 S seedlings per treatment and the experiment was repeated once. Data are presented as the means of both repetitions.

2.5 Analysis of free fatty acids

FG93R22 and SH430 caryopses were surface sterilized by shaking for 15 min in 8 g litre^{-1} sodium hypochlorite. After vigorous rinsing for 10 min in sterile distilled water, seeds were placed on one sheet of Whatman #4 filter paper in 25 × 100 mm Petri dishes moistened with 3 ml sterile distilled water. Petri dishes were sealed with Parafilm and incubated in the dark at 22(±2)°C until the seedlings were 1 cm long (about four days). Seedlings were treated by spotting 10 mg litre^{-1} or 100 mg litre^{-1} triallate (1 μl) or 100 mg litre^{-1} or 500 mg litre^{-1} triallate sulfoxide (1 μl) on the coleoptile tips using a Hamilton syringe. Control plants were treated with 70 g litre^{-1} surfactant in water. Five seedlings per treatment were harvested after 48 h and homogenized for 3 min in a Broeck glass homogenizer containing HPLC-grade methanol (0·5 ml). Methylene chloride (1 ml) was added and the mixture homogenized for an additional 1 min. The mixture was partitioned against two aliquots of distilled deionized water (0·5 ml) and the water/methanol fraction removed. An aliquot of the methylene chloride layer (0·25 ml) was removed, filtered to 0·22 μm through nylon microspin centrifuge filters and subjected to GC analysis.

C_{12} to C_{26} free fatty acids and fatty alcohols were derivatized with BSTFA as above and separated using a 30-m Alltech Carbowax capillary column in a HP Series II Plus gas chromatograph programmed to ramp from 175°C to 300°C at 5° min^{-1} using hydrogen as the carrier gas. Fatty acids and fatty alcohols were detected using flame ionization. Eluted peaks were identified based on coelution with known standards. There were two repetitions of eight seedlings for each treatment and peak area means from both repetitions are presented as percentages of untreated controls.

2.6 [^{14}C]Malonic acid incorporation

The effects of triallate and triallate sulfoxide on de-novo fatty acid biosynthesis were determined by comparing the incorporation of [2-^{14}C]malonic acid into lipid components in R and S seedlings. Four-day-old etiolated seedlings were treated with 10 mg litre^{-1} or 100 mg litre^{-1} triallate (1 μl) or 100 mg litre^{-1} or 500 mg litre^{-1} triallate sulfoxide solution (1 μl) on the coleoptile tips as described above. After 6 h, [2-^{14}C] malonic acid ($1{\cdot}67 \times 10^3$ Bq; sp. act. $1{\cdot}9 \times 10^7$ Bq mg^{-1}) dissolved in ethanol (1 μl) was spotted on the coleoptile tips, and five seedlings per treatment were harvested 48 h later and homogenized as above. The methylene chloride fraction was evaporated to dryness under vacuum and the extracts were resuspended in HPLC-grade hexane (0·5 ml) and filtered as above. Prior to HPLC analysis, samples were standardized to $6{\cdot}7 \times 10^2$ Bq by dilution in hexane. There were five repetitions for each treatment and the experiment was repeated once. Data are presented as the means of percentages of total radioactivity for individual peaks.

3 RESULTS

3.1 Epicuticular wax deposition

Two days after treatment, R and S seedlings treated with 0·1 kg ha^{-1} triallate exhibited a visible pinching

which marked the junction between leaf tissues present before treatment and new growth after treatment. Although triallate is typically applied as a pre-emergence soil-incorporated treatment, the post-emergence application provided a convenient method for comparing R and S tissue from the same leaves before and after exposure to the herbicide. Epicuticular wax deposition was visibly inhibited in S seedlings, as shown by a marked dulling and deep blue color of leaf tissues which grew after triallate application. Except for a similar pinching effect, R leaf tissues were not visibly affected by triallate treatment.

GC analysis was used to quantify and compare epicuticular wax deposition in R and S seedlings (Fig. 1). Wax was present on both R and S untreated leaf tissues at an average density of 34 μg cm^{-2}. However, triallate treatment caused a dramatic reduction in wax deposition in S seedlings. By five days after treatment, the quantity of surface wax on S wild oat leaves was reduced over 80% to 6 μg cm^{-2}. Epicuticular wax deposition on S wild oat leaves continued to be inhibited up to 21 days after treatment (data not shown). In contrast, wax deposition was not significantly reduced on R leaves. These data support earlier work in other species showing that formation of surface wax components was inhibited by thiocarbamate herbicides.[16–18] Mass spectroscopic analysis confirmed that more than 90% of the epicuticular wax on R and S wild oat leaves was the primary alcohol 1-hexacosanol ($C_{26}OH$; data not shown), as previously reported.[19] Triallate treatment did not affect the type of wax deposited on either R or S leaves (data not shown).

3.2 Analysis of free fatty acids

Triallate dramatically reduced pool sizes of several fatty acid constituents in etiolated S shoots (Table 1). At 10 mg litre^{-1} triallate, levels of C_{18}, C_{20} and C_{22} fatty acids and $C_{26}OH$ (1-hexacosanol) components were significantly reduced, with the greatest effect (72% reduction) in the last two fatty acids. All measured fatty acid constituents (except C_{12}) were reduced in S shoots by the 100 mg litre^{-1} triallate rate, and C_{22} and $C_{26}OH$ were undetectable. In contrast, fatty acid pool sizes were not affected in R shoots by either triallate rate tested. The data indicate that the biosynthesis of long-chain wax precursors is dramatically reduced in S by triallate treatment and lend support to the hypothesis that fatty acid elongation is a primary site of inhibition by triallate. However, since S plants sulfoxidize triallate much more rapidly than R plants,[3] these data do not address the relative importance of triallate sulfoxide in the herbicide's mechanism of action.

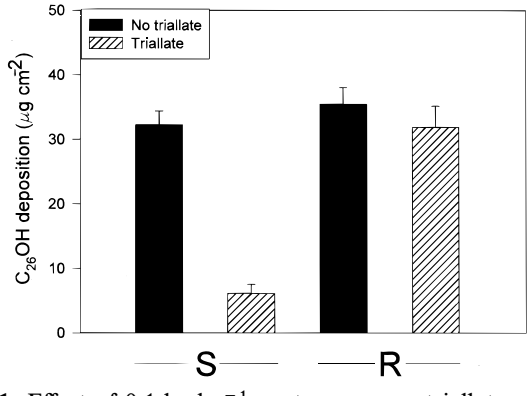

Fig. 1. Effect of 0·1 kg ha^{-1} post-emergence triallate on epicuticular wax synthesis in triallate-resistant (R) and susceptible (S) wild oat seedlings five days after treatment. Vertical bars are standard errors of means.

TABLE 1
Effect of Two Triallate Treatment Rates on in-vivo Concentrations of Fatty Acid Pools and One Alcohol in S and R Wild Oats 48 Hours after Treatment

	Triallate treatment rate (mg litre^{-1})			
	10		*100*	
Lipid	*S*	*R*	*S*	*R*
	Fatty acid pool sizes (% of untreated)[a,b]			
C_{12}	99	108	89	104
C_{16}	88	111	66*‡	100*
C_{18}	84‡	97	69‡	83
$C_{18:1}$	104	88	84‡	97
C_{20}	55*‡	106*	40*‡	90*
C_{22}	28*‡	94*	[c]n.d.*‡	101*
$C_{26}OH$	28*‡	96*	n.d.*‡	87*

[a] * significant difference between S and R at $P < 0{\cdot}05$.
[b] ‡ significant difference from untreated control at $P < 0{\cdot}05$.
[c] n.d. none detected.

Triallate sulfoxide also reduced fatty acid pool sizes in both R and S wild oat seedlings (Table 2). Due to the highly labile nature of triallate sulfoxide (half-life under these conditions of *c.*12 h, data not shown), we used higher treatment doses than for triallate. In S shoots, 100 mg litre^{-1} and 500 mg litre^{-1} triallate sulfoxide had the largest effect on long-chain fatty acids (C > 18), reducing pools 52–100% compared to untreated controls. However, C_{12} to $C_{18:1}$ fatty acid pools were not reduced. In R shoots, long-chain fatty acid pools were similarly reduced at both treatment levels, with the greatest effect seen in the C_{26} primary alcohol pool. The similar inhibition of long-chain fatty acid biosynthesis in S plants treated with triallate (Table 1) and triallate sulfoxide (Table 2) indicates that the two compounds have similar effects. However, the fact that fatty acid elongation was inhibited in R plants by triallate sulfoxide (Table 2) but not by triallate (Table 1) suggests that triallate sulfoxide is indeed the herbicidally active compound.

TABLE 2
Effect of Two Triallate Sulfoxide Treatment Rates on in-vivo Concentrations of Fatty Acid Pools and One Alcohol in S and R Wild Oats 48 Hours after Treatment

	Triallate sulfoxide treatment rate (mg litre^{-1})			
	100		*500*	
Lipid	*S*	*R*	*S*	*R*
	Fatty acid pool sizes (% of untreated)[a,b]			
C_{12}	88	94	81	96
C_{16}	92	99	74	98
C_{18}	86	97	77	93
$C_{18:1}$	97	104	101	103
C_{20}	48‡	64‡	49‡	52‡
C_{22}	5*‡	22*‡	[c]n.d.‡	12‡
C_{26}OH	n.d.‡	4‡	n.d.‡	5‡

[a] * significant difference between S and R at $P \leq 0{\cdot}05$.
[b] ‡ significant difference from untreated control at $P \leq 0{\cdot}05$.
[c] n.d. none detected.

3.3 De-novo fatty acid synthesis

To study the effects of triallate on de-novo fatty acid biosynthesis, we monitored the incorporation of [2-^{14}C]malonic acid into various fatty acids in R and S shoots (Table 3). In untreated controls, incorporation of radioactivity was similar between S and R, with approximately 32% of the ^{14}C accounted for in palmitate and progressively lesser amounts in C_{12}, C_{20} and C_{18} fatty acid pools. Treatment of S shoots with 10 mg litre^{-1} triallate had no significant effect on C_{12} or C_{16} fatty acid synthesis. However, biosynthesis of C_{20} fatty acids was inhibited by 75% and 96% at the 10 mg litre^{-1} and 100 mg litre^{-1} treatment rates, respectively. C_{18} pool sizes were slighly elevated in S shoots treated with 10 mg litre^{-1} triallate. These results agree closely with those of Abulnaja and Harwood,[7] who found that incorporation of [^{14}C]malonyl-CoA into C_{20}–C_{24} fatty acids was decreased 25–100% in germinating peas as triallate treatment rates increased, while incorporation of radioactivity into C_{16} and C_{18} fatty acids was slightly increased at all rates tested. In contrast to S shoots, fatty acid biosynthesis in R shoots was not affected by either triallate treatment rate tested. Thus, it appears that triallate treatment inhibits de-novo formation of long-chain fatty acids in S wild oats, but the same herbicide doses neither affect fatty acid synthesis nor induce herbicide injury symptoms in R plants. We propose that a significantly reduced rate of triallate sulfoxidation in R wild oats is responsible for the lack of response.

Triallate sulfoxide treatments reduced fatty acid biosynthesis in both R and S seedlings (Table 4). In S shoots, 100 mg litre^{-1} and 500 mg litre^{-1} triallate sulfoxide reduced biosynthesis of C_{20} fatty acid pools by 58% and 92%, respectively, while other fatty acid pools were not affected. Similarly, biosynthesis of C_{20} fatty acids in R shoots was reduced 82% and 73% at 100 mg litre^{-1} and 500 mg litre^{-1} triallate sulfoxide, respectively. Thus, long-chain fatty acid biosynthesis in both R and S wild oat seedlings is equally sensitive to triallate sulfoxide treatment.

This work supports earlier reports that long-chain fatty acid and wax biosynthesis, but not biosynthesis of C_{12} to C_{18} fatty acids, is a significant herbicidal target of triallate action. In addition, we confirm that triallate sulfoxide most likely causes this inhibition. Epicuticular wax is clearly disrupted in sensitive species, although it remains unclear if this perturbation alone is sufficient to cause plant death. More likely, the reduction of other fatty acid species with chain lengths greater than 20 may have significant impacts on cellular and organelle membranes. These results also support our work characterizing the mechanism of triallate resistance in wild oats as a decreased ability of R plants to sulfoxidize triallate to its more toxic metabolite.[3] The fact that fatty acid biosynthesis in R and S seedlings is equally sensitive to triallate sulfoxide strongly suggests that the

TABLE 3
Effect of Two Triallate Treatment Rates on Incorporation of [2-^{14}C] Malonic Acid into Fatty Acid Pools in S and R Wild Oats

	Triallate treatment rate (mg litre^{-1})					
	0		*10*		*100*	
Lipid	*S*	*R*	*S*	*R*	*S*	*R*
	Fatty acid pool sizes (% of extractable radioactivity)[a]					
C_{12}	18	17	16	16	16	16
C_{16}	33	31	33	33	28*	33
C_{18}	5	5	7*	5	4	4
C_{20}	12	11	3*	13	0·5*	10

[a] * significant difference from untreated control at $P \leq 0{\cdot}05$.

TABLE 4
Effect of Two Triallate Sulfoxide Treatment Rates on Incorporation of [2-^{14}C] Malonic Acid into Fatty Acid Pools in S and R Wild Oats

	Triallate sulfoxide treatment rate (mg litre^{-1})					
	0		*100*		*500*	
Lipid	*S*	*R*	*S*	*R*	*S*	*R*
	Fatty acid pool sizes (% of extractable radioactivity)[a]					
C_{12}	18	17	17	20	20	18
C_{16}	33	31	32	31	30	28
C_{18}	5	5	6	6	5	6
C_{20}	12	11	5*	2*	1*	3*

[a] * significant difference from untreated control at $P \leq 0{\cdot}05$.

mechanism of resistance does not involve an alteration in elongase activity or sensitivity, but rather that R plants are avoiding high in-vivo concentrations of triallate sulfoxide and thus escaping its herbicidal effects. The use of triallate-resistant plants provides compelling evidence that in-vivo sulfoxidation is required for the herbicide to exert phytotoxic effects in wild oat seedlings.

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